

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 561 172 A1

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: **93102626.4**

(51) Int. Cl.⁵: **C12N 15/12, C12Q 1/68**

(22) Date of filing: **23.09.87**

This application was filed on 19 - 02 - 1993 as a divisional application to the application mentioned under INID code 60.

(30) Priority: **26.09.86 US 911863**

(43) Date of publication of application:
22.09.93 Bulletin 93/38

(60) Publication number of the earlier application in accordance with Art.76 EPC: **0 324 788**

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

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(54) **Recombinant DNA Clone Encoding Laminin Receptor.**

(57) A recombinant cDNA clone encoding a cell matrix receptor specific for laminin which is expressed on the surface of carcinoma and epithelial cells is provided, from which hybridisation probes are useful in cancer diagnosis.

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BACKGROUND OF THE INVENTION

Technical Field

5 The present invention is related generally to recombinant DNA. More particularly, the present invention is related to a recombinant cDNA clone encoding high affinity (about 10^{-8} to 10^{-10} Kd) cell surface receptors for laminin.

State of the Art

10 Laminin is a major glycoprotein component of basement membranes and mediates the attachment of both epithelial and neoplastic cells to the basement membrane. The basement membrane is a ubiquitous, specialized type of extracellular matrix separating organ parenchymal cells from interstitial collagenous stroma. Interaction of cells with this matrix is an important aspect of both normal and neoplastic cellular
15 processes. Normal cells appear to require an extracellular matrix for survival, proliferation, and differentiation, while migratory cells, both normal and neoplastic, must traverse the basement membrane in moving from one tissue to another. In particular, metastatic cancer cells arising in squamous or glandular epithelium must traverse the basement membrane to enter the circulatory and lymphatic systems (intravasation); the circulating neoplastic cells are typically arrested in the capillary beds of an organ, invade the blood vessel
20 walls, and penetrate the basement membrane to extravascular tissue (extravasation), where a secondary neoplasm is then established. Laminin receptor, by mediating the attachment of both epithelial and neoplastic cells to the basement membrane, plays a critical role in controlling, *inter alia*, the metastatic process. U.S. Patent 4,565,789 describes the isolation and characterization of certain aspects of laminin receptor. But a cloned DNA sequence for encoding cell surface receptor for laminin has not heretofore been
25 known.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a recombinant cDNA clone capable of
30 encoding high affinity (about 10^{-8} to 10^{-10} Kd) cell surface receptor for laminin.

It is an additional object of the present invention to provide synthetic fragments of laminin receptor which encode specific domains such as membrane-spanning and ligand binding regions.

It is another object of the present invention to provide a diagnostic method to assess the content of laminin receptor mRNA in different tumor cell and normal tissue cell populations.

35 It is a still further object of the present invention to provide a diagnostic method to determine the pattern of laminin receptor genes in different tissue and tumor cell populations.

BRIEF DESCRIPTION OF DRAWINGS

40 These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

FIG. 1 demonstrates the homogeneity of purified laminin receptor. TOP shows comparison of the purified laminin receptor from colon carcinoma and from normal human placental tissue. Materials eluted with 1
45 M NaCl from the laminin-Sepharose affinity column were analyzed by NaDodSO₄-polyacrylamide gel electrophoresis (7%) in the presence of dithiothreitol and visualized with silver staining. The panel exemplifies the slight variability in the molecular weight (68,000-72,000) between preparations of the laminin receptor. Lane P1: Laminin receptor (1 μ g) isolated from human placental tissue. Lane Ca: Laminin receptor (3 μ g) isolated from human colon carcinoma. Lane M: Molecular weight markers:
50 phosphorylase b (94,000), bovine serum albumin (67,000), and ovalbumin (43,000).

BOTTOM shows isoelectric focusing and two-dimensional gel electrophoresis of the purified laminin receptor. The colon carcinoma laminin receptor protein sample (0.1 μ g) shown above was radiolabeled with I¹²⁵ and subjected to isoelectric focusing (pH range 5-7) and two-dimensional gel electrophoresis on a 10% NaDodSO₄-polyacrylamide gel. Molecular weight markers: phosphorylase b (93,000), bovine
55 serum albumin (66,000), glyceraldehyde 3-phosphate dehydrogenase (36,000);

FIG. 2 shows an ELISA of the purified laminin receptor. Each microtiter well was coated with purified tumor laminin receptor and reacted for 2 hours against LR1 monoclonal antibody 2H5 ($\square$), anti-albumin (O) or anti- α -amylase monoclonal antibody ($\bullet$). Antibodies were detected using peroxidase-conjugated

goat anti-mouse IgM or peroxidase-conjugated swine anti-rabbit IgG;

FIG. 3 depicts the specificity of the anti-laminin receptor monoclonal antibody for λ ELR6 plaques. Filters containing approximately 50-100 purified λ ELR6 plaques were reacted with either anti-laminin receptor monoclonal antibody, anti- α -amylase monoclonal antibody, anti-albumin antibody or anti-laminin antibody. Antibody binding was detected using peroxidase-conjugated second antibody;

FIG. 4 demonstrates the overlapping laminin receptor cDNA inserts of λ ELR1-6. The top line is a diagram of prototype λ ELR recombinant λ gt11 phage, showing the two KpnI sites (K) to the left of the cDNA inserts. The box depicts the cDNA insert. The left EcoRI site in each insert was not present. The right EcoRI site (E) is shown. Transcription from the *lacZ* gene of λ gt11 proceeds from the right arm of the phage toward the left, through E. The region extending from the KpnI site (closest to the cDNA insert) to the EcoRI site of the largest cDNA insert (λ ELR4) is expanded and shown below. The stippled area in the cDNA insert box designates the PstI-SphI restriction fragment isolated from a subclone of λ ELR4 (see FIG. 5) which was nick translated and used as a hybridization probe. The common SacI (S) and HindIII (H) sites described in the text are indicated. Phage DNA from each of the ELR recombinant phage was restricted with KpnI and EcoRI, electrophoresed through a 1% agarose gel, transferred to nitrocellulose, and stringently hybridized to the PstI-SphI restriction fragment described above. The length of the KpnI-EcoRI restriction fragment from each of the λ ELR recombinant phage which hybridized to the probe is diagrammed above the radioautograph of the blot, and was determined by comparison to the standard λ /HindIII digests;

FIG. 5 diagrams the strategy to sequence the cDNA insert of λ ELR4. The restriction map of subcloned insert of λ ELR4 is shown on top. E is the 5' EcoRI site of the insert. H: HinfI, S: SphI, R: RsaI, P: PstI, *: ochre termination codon. The arrows indicate the sequencing direction of fragments labeled either by kinasing at the 5' end with (γ - P^{32}) ATP (●) or by tailing at the 3' end with (α - P^{32}) dideoxy ATP (O) or by the dideoxy synthesis method (r). Subclones used for sequencing were pLR4-1, pLR4-2, and pLR4-4;

FIG. 6 provides the nucleotide sequence of the λ ELR cDNA insert with derived protein sequence below. Shown to the right of each line is the number of base pairs from the artificial EcoRI site (bases 1-6);

FIG. 7 shows ELISA determination of synthetic laminin receptor peptide. Synthetic laminin receptor peptide RTLR2 was generated from the cDNA sequence in FIG. 6 and was assayed by ELISA using different dilutions of rabbit anti-laminin receptor antiserum or perimmune serum;

FIG. 8 shows an RNA gel blot hybridization. Total cellular RNA was extracted from several breast carcinoma cell lines, separated on methylmercury agarose gels and transferred to diazobenzylxymethylcellulose paper. The filter was hybridized to nick translated (32 P)-labeled EcoRI-PstI insert from pLR4-1. The length of the hybridized mRNA species was determined by comparison with the known sizes of rRNA and with the sizes of λ DNA restricted with HindIII. Lane 1: MCF-7 (parent); Lane 2: MCF7-3E5; Lane 3: MCF7-5H7; Lane 4: ZR75;

FIG. 9 shows the correlation of laminin receptor mRNA levels with ability of six human carcinoma cell lines to bind laminin. Different timed exposures of radioautographs such as shown in FIG. 8 were measured densitometrically to determine a linear response range. The lowest amount of RNA hybridized in the series was assigned the number 1.0 and all other values were calculated relative to that value. The number of laminin receptors per cell for each cell line was calculated from Scatchard plots of specifically bound (I^{125}) laminin to log growth phase cells. A linear regression analysis revealed a correlation coefficient of 0.97. MCF-7 parent □, MCF-7 Clone 5H7 △, MCF-7 Clone 3E5 ■, ZR-75 O, renal carcinoma A-704 ●, Panc-1 ▲;

FIG. 10 demonstrates that synthetic laminin receptor peptide inhibits laminin binding to cells. The ability of A2058 human melanoma cells to bind specifically to laminin was tested in the presence of various amount of RTLR2 □ or RTLR2-control ◆ fragments generated from the cDNA sequence of FIG. 6. Values are expressed relative to the amount of laminin bound in the absence of added fragment; and

FIG. 11 shows identification of synthetic peptide laminin receptor fragments involved in ligand binding. Peptide fragments generated from cDNA sequence with predicted reverse turn conformation (RTLR2, RTLR3) as well as control peptides were bound to glass beads and incubated with (I^{125}) laminin. The values shown are the number of cpm $\times 10^{-3}$ bound to beads after subtraction of cpm bound to beads without fragment.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved in accordance with Claims 1 to 8.

Unless defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

The methodology for constructing and isolating the recombinant cDNA clone of Figure 6 is described in further detail in Example II and includes the following general steps, involving standard techniques well known in the art:

- a) cDNA is synthesized using mRNA template from human umbilical vein endothelial cells.
- b) Endothelial cell cDNA is inserted into the λ gt11 vector (ATCC 37194).
- c) The recombinant λ gt11 is packaged and used to infect *Escherichia coli* 1090 cells (ATCC 37197).
- d) The resulting λ gt11 human endothelial cell cDNA expression library is screened with a monoclonal antibody which recognizes a domain of the human laminin receptor involved in binding of laminin.
- e) Six plaques, designated λ ELR1-6, are thus isolated and purified (FIG. 3). The cDNA inserts are analyzed by restriction endonuclease mapping and are found to have a common domain, indicating that they encode the epitope of the laminin receptor recognized by the monoclonal antibody (FIG. 4).
- f) The largest cDNA insert of the six purified phase is subcloned into plasmid vectors to facilitate further analysis and DNA sequencing. The EcoRI site linking the cDNA insert to the left arm of λ gt11 is missing in all six clones. A PvuII site 11 bp downstream is therefore utilized to purify cDNA insert from the largest recombinant phage, λ ELR4. The EcoRI-PvuII restriction fragment of λ ELR4 is subcloned into the EcoRI-PvuII sites of pBR322 to make pLR4-1.

A deposit of the recombinant cDNA clone pLR4-1 transformed into host *E. coli* strain C600r⁻m⁻ (ATCC 33525) obtained in accordance with the present invention has been made at the American Type Culture Collection (ATCC), Rockville, MD, under accession number 67199. Upon request, the Commissioner of the PTO shall have access to the deposit, and upon issuance of the patent, this deposit will continue to be viably maintained for at least 5 years after the last request or at least 30 years or the life of the patent and made available to the public without restriction, of course, consistent with the provisions of the law.

The method for definitively identifying the present invention was based on comparison of cDNA sequence of the recombinant plasmids (FIG. 6) with the amino acid sequence of a cyanogen bromide-generated octapeptide of purified placental laminin receptor. The methodology for obtaining octapeptide amino acid sequence is described in further detail in Example I and includes the following general steps, involving standard techniques well known in the art:

- a) Microsomal membranes from human placental tissue are prepared and solubilized.
- b) The solubilized microsomal membrane-containing preparation is passed through an affinity matrix of purified laminin-Sepharose 4B and after extensive washing, the high affinity laminin receptor protein is eluted from the ligand by high salt (1 M NaCl).
- c) The homogeneity of the human placental laminin receptor is assayed by two dimensional gel electrophoresis (FIG. 1) and enzyme-linked immunosorbent assay (ELISA) (FIG. 2).
- d) The human placental laminin receptor is cleaved with cyanogen bromide and the resulting peptides are fractionated by reverse phase high pressure liquid chromatography (HPLC).
- e) Microsequence analysis of a placental oligopeptide reveals an octapeptide with the unique sequence Met-Leu-Ala-Arg-Glu-Val-Leu-Arg.

An alternate method for identifying the present invention was based on the ability of rabbit antiserum directed again human metastatic breast carcinoma laminin receptor to identify (by ELISA), a synthetic peptide generated from the cDNA sequence of pLR4-1 (FIG. 7). The methodology for obtaining the anti-laminin receptor antiserum is described in further detail in Example IV and includes the following general steps, involving standard techniques well known in the art:

- a) Laminin receptor is purified from human metastatic breast carcinoma as described supra for placental laminin receptor.
- b) Homogeneity of the tumor laminin receptor is assessed as described supra for placental laminin receptor.
- c) Rabbits are immunized by subcutaneous injections of tumor laminin receptor cut out of NaDodSO₄-polyacrylamide gels.

Diagnostic assays for cells containing laminin receptor mRNA, such as human cancer cells, have been developed as useful tools in the diagnosis and prognosis of cancer. The method for assaying laminin receptor mRNA content is described in further detail in Example V and includes the following general steps, following standard techniques well known in the art:

- a) Total cellular RNA is isolated from tissue specimen or cells.

b) RNA is denatured and electrophoresed through denaturing agarose gels and transferred to a filter paper.

c) The filter paper containing the RNA is hybridized to radiolabeled laminin receptor cDNA.

d) After washing, the filter is exposed to X-ray film for sufficient time to obtain an image of the radioactive probe.

e) The relative intensity of the band on the film is a measure of the content of laminin receptor mRNA (FIG. 8).

f) Alternatively, in lieu of step (b) above, a small aliquot of denatured RNA can be directly bound to filter paper followed by steps (c) thru (e).

g) Radiolabeled laminin receptor cDNA can also be hybridized in situ to tissue sections and cells to determine specific cell populations expressing laminin receptor mRNA.

Increased content of laminin receptor mRNA in metastatic cells lends to the development of diagnostic assays for cells containing altered laminin receptor DNA. The methodology is based on the possible presence of restriction fragment length polymorphisms as has been found for a variety of genetic diseases. Amplification of laminin receptor genes in the genome of tumor or metastatic cells may also be assessed. The method for analyzing laminin receptor genomic DNA includes the following general steps, following standard techniques well known in the art:

a) High molecular weight genomic DNA is isolated from tissue specimen or cells, as described in Example VI.

b) DNA is digested with a variety of restriction endonucleases, electrophoresed through agarose gel and transferred to filter paper.

c) The filter containing the DNA is hybridized to radiolabeled laminin receptor cDNA.

d) After washing, the filter is exposed to X-ray film and the pattern of bands on the film is compared to that from normal tissue.

The methods described herein are based on the observation that the basement membrane is a continuous structure in benign neoplasms, and that laminin receptors on benign cells are occupied by attachment to basement membrane. In contrast, the basement membrane adjacent to invading tumor cells is not continuous. Invading tumor cells thus may have increased numbers of laminin receptors expressed on the cell surface which are not bound to ligand. It is the availability of laminin receptor sites on metastasizing carcinoma cells which is an important concept in the management and diagnosis of cancer. The aggressiveness of a carcinoma, defined as the tendency for a tumor cell of that carcinoma to migrate out of the primary tumor site into adjacent normal tissue and its propensity for spreading to distant sites in the body to initiate metastatic clones, is, then, in part, a reflection of the number of invasive cells with more available laminin receptors.

Based on the above, it is clear that the present invention can be used in a variety of ways in the diagnosis of cancer, as well as other diseases resulting from abnormal recognition of laminin by cells.

Specific examples to illustrate the present invention are now described.

EXAMPLE I

Isolation of Human Laminin Receptor cDNA Clones

A. Materials and Methods

1. Cell Culture - Monolayer cultures of human umbilical vein endothelial cells were grown as described by Jaye, et al., *Science*, 228:882-885 (1985).

2. Preparation of RNA - Total cellular RNA was extracted from cell layers in culture by the guanidinium isothiocyanate-caesium chloride density method described by Chirgwin, et al., *Biochemistry*, 18:5294-5299 (1979). Poly (A)-containing RNA was isolated by chromatography on oligo(dT) cellulose (Type 3, Collaborative Research) according to the method of Aviv and Leder, *Proc. Natl. Acad. Sci.*, 69:1408-1412 (1972).

3. Synthesis of cDNA - Oligo(dT)-selected human endothelial RNA was used as template for the synthesis of cDNA by the method of Buell, et al., *J. Biol. Chem.*, 253:2471-2482 (1978) and double stranded cDNA was prepared as described by Wickens, et al., *J. Biol. Chem.*, 253:2483-2495 (1978). The cDNA was made blunt ended with S1 nuclease as described by Ullrich, et al., *Science*, 196:1313-1319 (1977) and subsequent treatment with the Klenow fragment of *E. coli* DNA polymerase I as described by Wartell and Resnikoff, *Gene*, 9:309-319 (1980). The cDNA was then modified with EcoRI methylase, ligated to EcoRI linkers and exhaustively digested with EcoRI as described by Huynh, et al., *DNA*

Cloning Volume 1: a practical approach, ed., D.M. Glover, IRL Press Ltd., Oxford, England, pp. 49-78 (1985).

4. Construction and Screening of Human Endothelial λ gt11 cDNA Library - EcoRI-linked cDNA was ligated to EcoRI-digested and phosphatase-treated λ gt11 as described by Huynh (1985) *supra*. The recombinant phage were packaged as described by Enquist and Sternberg, *Methods Enzymol.*, 68:281-298 (1979) and amplified in *E. coli* Y1088 (ATCC 37195) as described by Huynh (1985) *supra*. *E. coli* Y1090 (ATCC 3/19) cells were infected with the endothelial cell λ gt11 cDNA library and 1.5 million plaques were screened by antibody recognition of a laminin receptor β -galactosidase fusion protein, as described by Young and Davis, *Science*, 222:778-782 (1983), using anti-laminin receptor monoclonal antibody 2H5 (Liotta, et al., *Exp. Cell Res.*, 156:117-126 (1985). This antibody recognizes or interferes with the ligand binding of the laminin receptor (Liotta, et al. (1985) *supra* and Togo, et al., *Basement Membranes*, ed. Shibata, S., Elsevier, New York, pp. 325-333 (1985)). Antibody binding was detected using peroxidase-conjugated affinity-purified rabbit anti-mouse IgM as described *supra* in Example 1A4. Positive plaques were identified, amplified, and rescreened to purity as described by Benton and Davis, *Science*, 196:180-182. Filters containing plaques were reacted against control antibodies, including anti-human α -amylase and anti-human albumin (as described in Example 1A4 *supra*) as well as anti-rat laminin (Albrechtsen, et al., *Cancer Res.*, 41:5076-5081 (1981)).

5. Southern Blot Hybridization - Phage DNA was isolated as described by Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 77-85 (1982). Restriction endonuclease-digested DNAs were electrophoresed through agarose gels, transferred to nitrocellulose paper, and hybridized by the method of Southern, *Methods Enzymol.*, 68:152-176 (1979). 32 P-labeled probes were prepared by nick translation as described by Maniatis, et al., *Proc. Natl. Acad. Sci.*, 72:1184-1188 (1975).

6. Subcloning and Preparation of Plasmids and cDNA Insert - The EcoRI site linking the cDNA insert to the left arm of λ gt11 was missing in all six clones. A PvuII site 11 bp downstream was therefore utilized to purify cDNA insert from the largest recombinant phage ELR4. The EcoRI-PvuII restriction fragment of λ ELR4 was subcloned into the EcoRI-PvuII sites of pBR322 to make pLR4-1 and into the EcoRI-HindIII polylinker sites of pUC8 to make pLR4-2. Subsequently, to facilitate cDNA sequencing away from the long poly(A) tail, the EcoRI-PstI restriction fragment of pLR4-1 was subcloned into the EcoRI-PstI sites of pBR322 to make pLR4-4. Recombinants of pBR322 were transformed into *E. coli* C600r m (ATCC 33525) and pUC recombinants were transformed into *E. coli* JM 103 (Messing, et al., *Nucleic Acids Res.*, 9:309-321 by the calcium chloride procedure of Mandel and Higa, *J. Mol. Biol.*, 53:159-162 (1970)). Supercoiled plasmid DNA was recovered by alkali lysis after equilibrium centrifugation in ethidium bromide-cesium chloride according to the procedures of Birnboim and Doly, *Nucleic Acids Res.*, 7:1513-1523 (1979). cDNA inserts were isolated from restricted DNA and eluted from acrylamide gels as described by Sobel, et al., *Proc. Natl. Sci.*, 75:5846-5850 (1978).

B. Results

1. Selection of Laminin Receptor cDNA by Antibody Recognition - The human endothelial cell λ gt11 cDNA library was screened using anti-laminin receptor monoclonal antibody 2H5. As described in Materials and Methods, *supra*, this antibody (a) specifically recognizes the laminin receptor on immunoblots, (b) recognizes the purified laminin receptor by ELISA (FIG. 2), (c) blocks the binding of laminin to either plasma membranes or cells, and (d) blocks attachment of cells to amnion basement membrane. These properties clearly indicate that the monoclonal antibody recognizes or interferes with the laminin binding domain of the laminin receptor. Six plaques were initially selected. After plaque purification, all six phage designated λ ELR1-6, showed an intense reaction with the anti-laminin receptor monoclonal antibody, but showed no reactivity toward a class matched (IgM) monoclonal antibody directed against human α -amylase, nor toward antibodies directed against laminin or against proteins similar in size to laminin receptor such as albumin (FIG. 3).

2. Common Domain of λ ELR1-6 - Restriction endonuclease digestion of the six phage, λ ELR1-6, revealed that the EcoRI site linking the 3' end of the cDNA insert to the left arm of λ gt11 was missing in all six phage. Single, double, and triple endonuclease digestions using SacI, Kpl, HindIII, and EcoRI were performed to map λ ELR1-6. These experiments, summarized in FIG. 4, indicated that (a) the sizes of the cDNA inserts ranged from about 450 to 975 bp, (b) all the clones shared an internal SacI site approximately 450-500 bp from the 3' end of the clones, (c) the insert of the λ ELR6 extended only about 20 bp 5' to the internal SacI site, and (d) the inserts of λ ELR1 and λ ELR4 shared a HindIII site approximately 30 and 40 bp, respectively, from the 5' EcoRI site. The six recombinant phage thus have

a common 450-500 bp region, extending from just 5' to the SacI site to the 3' end of each cDNA insert, suggesting that this region encodes the domain recognized by the monoclonal antibody 2H5. This observation was tested by Southern blot experiments in which KpnI-EcoRI and KpnI-SacI double digests of the DNA from each of the six recombinant phage were stringently hybridized to a PstI-SphI restriction fragment isolated from λ ELR4. A representative hybridization experiment, shown in FIG. 4, shows that a common 450-500 bp region of all six recombinant phage encodes the antigenic domain recognized by monoclonal antibody 2H5.

EXAMPLE II

cDNA Sequence Determination

A. Materials and Methods

1. DNA Sequencing - The cDNA from λ ELR4 was sequenced according to the strategy shown in FIG. 5. Both the chemical modification method of Gilbert and Maxam, *Proc. Natl. Acad. Sci.*, 70:3581-3584 (1973) were used. In the former case, cDNA restriction fragments of subclones pLR4-1, pLR4-2, and pLR4-4 described in Example IIA6, supra, were labeled either by treating the 5' end with T4 polynucleotide kinase (Boehringer-Mannheim) and (γ - P^{32})ATP as described by Gilbert and Maxam (1973), supra, or by tailing the 3' end with (α - P^{32}) dideoxy ATP using a 3' tailing kit (Amersham). In the case of the dideoxy synthesis method, the EcoRI-SacI restriction fragment of ELR4 was subcloned into M13mp18 and M13mp19 (Yanisch-Perran, et al., *Gene*, 33:103-119 (1981)).

B. Results

1. Nucleotide and Deduced Amino Acid Sequence of the cDNA insert of λ ELR4 - Sequence of the entire cDNA insert of λ ELR4 reveals a 253 amino acid open reading frame consistent with the right arm EcoRI insertion site of the λ gt11 vector (FIG. 6). The sequence of the "common domain" recognized by the 2H5 epitope (see Example IIB2, supra) extends from nucleotide 361 to nucleotide 765 in FIG. 6. It includes an octapeptide-encoding sequence (nucleotides 409-432) identical to that of the purified cyanogen bromide-generated oligopeptide of placental laminin receptor described in Example Ib2, supra. Notable within this sequence is a six amino acid repeat separated by 3 amino acids toward the carboxy terminus of the laminin receptor. The cDNA sequences which encode the amino acid repeats are not identical (nucleotides 670-687 and 697-714). Following an ochre stop codon, the λ ELR4 cDNA insert includes a 3' untranslated region of 66 bp, including a canonical polyadenylation signal AATAAA 17 bp upstream from a long poly(A) stretch. After subtracting the length of the 3' untranslated region and the poly(A) tail, the largest possible extent of the 2H5 epitope, as defined by the common cDNA region of λ ELR1-6, can be narrowed down to 393 bp or 131 amino acids. Computer analysis by the method of Wilbur and Lipman, *Proc. Natl. Acad. Sci.*, 80: 726-730 (1983) reveals no significant homologies to known protein sequences.

It is noted that a potential membrane-spanning domain of the laminin receptor could include an eleven amino acid hydrophobic polypeptide encoded by nucleotides 52-84 of FIG. 6.

EXAMPLE III

Hybridization of Laminin Receptor cDNA to RNA

A. Materials and Methods

1. Cell Lines - The MCF-7 human breast cancer cell line (parent) was provided by the Michigan Cancer Foundation. Two cloned MCF-7 cell lines MCF7-5H& and MCF7-3E5, were obtained from P. Horan Hand (National Cancer Institute) and are described by Greiner, et al., *Int. J. Cancer*, 36:159-166 (1985). The ZR-75 human breast carcinoma line is described by Engel and Young, *Cancer Res.*, 38:4327-4339 (1978) and was obtained from L. Engel (National Cancer Institute) and the human renal carcinoma line A-704 was from S. Aaronson (National Cancer Institute) and is described by Giard, et al., *J. Natl. Cancer Inst.*, 51:1417-1423 (1973). The human Panc-1 cell line was obtained from the American Type Culture Collection. Murine NIH-3T3 cells and its Kirsten virus-transformed derivative (KNIH) were obtained from M. Gottesman (National Cancer Institute) and their growth is described by Gottesman, *Proc. Natl. Acad.*

Sci., 75:27657-2771 (1978). Murine F9 teratocarcinoma cells and their dibutyrylcyclic AMP plus retinoic acid differentiated derivatives (Strickland, et al., Cell, 21:347-355 (1980)) were from W. Anderson (National Cancer Institute). The rat L2 cell line is described by Wewer, Dev. Biol., 93: 416-421 (1982).

2. Preparation of RNA - RNA was prepared from cell cultures as described in Example IA2, *supra*, or by the guanidine-hydrochloride method described by Strohmman, et al., Cell, 10:265-273 (1977).

3. Northern Hybridization - 5 µg of total cellular RNA were separated on methylmercury/agarose gels and transferred to diazobenzylxymethylcellulose paper (Schleicher and Schuell) by the method of Alwine, et al., Proc. Natl. Acad. Sci., 74:5350-5354 (1977) as described by Sobel, et al., Biochemistry, 20:2678-2684 (1981). Filters were hybridized to nick translated cDNA insert as described in Example IA5, *supra*. Various time exposures of radioautographs were measured densitometrically to determine linear-response range. The blots were counter-screened with actin cDNA probe (described by Cleveland, et al., Cell, 20:95-105 (1980)) and with heat shock cDNA probe (described by Hickey, et al. Gene, 43:147-154 (1986)) to ensure equal amounts of RNA were transferred.

4. Laminin-Binding Assays - The number of laminin receptors per cell for each cell line was calculated from Scatchard plots of specifically bound (¹²⁵I)-labeled laminin to logarithmic growth phase cells as described by Liotta, et al., Exp. Cell Res., 156:117-126 (1985).

B. Results

1. Expression of Human Laminin Receptor mRNA -It was previously reported that the amount and surface distribution of laminin receptor is different in various carcinomatous human tissues (Horan Hand, et al., Cancer Res., 45:2713-2719 (1985)). In general, malignant tissues have more unoccupied laminin receptors on their cell surface and bind to more laminin than do their more benign counterparts. To determine if laminin receptor mRNA levels play a role in determining the amount of cell surface laminin receptors available for ligand binding, a Northern blot experiment was performed (FIG. 8). The laminin receptor cDNA insert recognizes a mRNA of about 1700 bases which is sufficient in length to encode a protein with the estimated size of laminin receptor. The level of hybridized RNA from a variety of human epithelial cell lines varied. In particular, there was greater hybridization to RNA from the metastatic breast carcinoma cell line MCF-7 than to RNA from the nonmetastatic breast carcinoma cell line ZR-75. In general, the level of hybridized RNA correlated directly with laminin binding assays (Fig. 9), suggesting that the amount of laminin receptor mRNA available for the biosynthesis of receptor may be a rate-limiting control step in the regulation of cellular attachment to laminin the basement membrane. Hence, the determination of laminin receptor mRNA levels in human tumor tissue can be used diagnostically to predict the relative aggressiveness of the tumor or susceptibility to therapeutic regimens based on laminin receptor content. As mentioned above, this can be accomplished by Northern blot hybridization or in situ hybridization of sectioned tumor material, using laminin receptor cDNA as probe. Laminin receptor cDNA may be prepared as probe for the above mentioned procedures by a variety of means following standard techniques well known in the art. The nick translation procedure described in Example IIA5 can be used to radiolabel cDNA insert with either (³²P), (³⁵S), or (³H). Alternatively, the laminin receptor cDNA insert can be subcloned into plasmids permitting transcription of radiolabeled RNA by methods described by Melton, et al., Nucleic Acids Res., 12:7035-7056 (1984). Biotinylated RNA and DNA hybridization probes in conjunction with chromogenic detection systems have also been described, for example, by Langer, et al., Proc. Natl. Acad. Sci., 78:6633-6637 (1981) and by Leary, et al., Proc. Natl. Acad. Sci., 80:4045-4049 (1983).

2. Detection of Laminin Receptor mRNA in Other Species - Northern blots containing RNA from rat L2 cells and from murine cells such as NIH 3T3 and F9 teratocarcinoma and their derivatives also detected a mRNA of about 1700 bases. Thus, the human laminin receptor cDNA insert can cross hybridize to RNA from other vertebrate species (data not shown).

EXAMPLE IV

Polymorphisms of Laminin Receptor

A. Materials and Methods

1. Identification of Cross-Hybridizing Laminin Receptor cDNAs - The human endothelial cell λgt11 cDNA library described in Example IA4 was used to infect E. coli Y1088 cells (ATCC 37195) and screened using ³²P-labeled cDNA insert from λELR4 by the plaque hybridization procedure of Benton and Davis,

Science, 196:180-182 (1977). Positive plaques were identified amplified, and rescreened to purity as described by Benton and Davis, *supra*.

2. DNA Sequence Determination - The cDNA inserts of positive phage here subcloned into the EcoRI site of pUC vectors as described in Example IA6, and cDNA sequence was determined by the method of Gilbert and Maxam, *Proc. Natl. Acad. Sci.*, 70:3581-3584 (1973).

3. Isolation of Human Genomic DNA - Genomic DNA was isolated from human cell lines described in Example IIIA1 *supra* by a gentle digestion with sodium dodecylsulfate and proteinase K as described by Blin, et al., *Nucleic Acids Res.*, 3:2303-2308 (1976).

4. Southern Hybridizations - Genomic DNA from the cell lines described above as well as from human liver (obtained from M. Young, National Institute of Dental Research) was digested exhaustively with EcoRI, HindIII, BamHI, or XbaI, electrophoresed on agarose gels, transferred to nitrocellulose and hybridized to λ ELR4 cDNA insert as described in Example IIA5, *supra*.

B. Results

1. cDNA Sequence Comparison - Partial cDNA sequences were determined for recombinant phage λ ELR10, λ ELR14, λ ELR106, λ ELR112. The sequence from the 5' EcoRI site to the internal SacI site of λ ELR14 completely corresponds to nucleotides 7-381 of FIG. 6 and includes 96 additional bases upstream from λ ELR4. The other clones analyzed show high homology to λ ELR4 sequences but contain multiple point mutations. For each of the other clones, there are no open translational reading frames, suggesting that they may be cDNAs of pseudolaminin receptor genes.

2. Genomic DNA Analysis - Southern blot analysis of high molecular weight genomic DNA from human liver and from a variety of human cell lines shows multiple hybridizing DNA bands. This suggests that there are multiple laminin receptor genes in the human genome, all of which cross-hybridize. In addition, multiple plaques, each containing unique DNA, were isolated by the plaque hybridization procedure described above from a λ Charon 4A library (obtained from T. Maniatis, Harvard University) of human genomic DNA partially digested with AluI/HaeIII. These findings suggest that the multiple cross-hybridizing laminin receptor genes are not identical. One possibility is that the human genome contains one active laminin receptor gene and several pseudogenes. The mRNAs transcribed from the pseudogenes are sufficiently stable to reach the cytoplasm and can be transcribed in vitro to make cDNA, and their corresponding cDNAs cross-hybridize with authentic laminin receptor cDNA such as λ ELR1-6 and can be used as alternative hybridization probes in Northern blots. However, due to multiple translational stop codons at the 5' ends of the pseudogenes, laminin receptor protein is not synthesized from them. It is necessary that all isolated laminin receptor cDNAs encode (in open reading frame) the amino acids predicted by λ ELR4 cDNA sequence to qualify as authentic laminin receptor cDNA.

Claims

1. A method for diagnosing the aggressiveness of a carcinoma or the effectiveness of an agent for treating cancer cells comprising reacting a sample of a tissue suspected to have said cancer cells with a probe, the diagnosis depending on the degree of hybridisation, characterised in that the probe is obtained from a recombinant cDNA clone encoding cell surface receptor for laminin.
2. A method according to Claim 1, wherein said clone has a sequence corresponding to the cDNA contained in ATCC 67199 encoding laminin cell surface receptor.
3. A method according to Claim 1, wherein said clone has the following sequence:

CGT GAT GGC ATC TAT ATC ATA AAT CTC AAG AGG ACC TGG GAG AAG
 CTT CTG CTG GCA GCT CGT GCA ATT GTT GCC ATT GAA AAC CCT GCT
 GAT GTC AGT GTT ATA TCC TCC AGG AAT ACT GGC CAG AGG GCT GTG
 5 CTG AAG TTT GCT GCT GCC ACT GGA GCC ACT CCA ATT GCT GGC CGC
 TTC ACT CCT GGA ACC TTC ACT AAC CAG ATC CAG GCA GCC TTC CGG
 AAG CCA CGG CTT CTT GTG GTT ACT GAC CCC AGG GCT GAC CAC CAG
 10 CCT CTC ACG GAG GCA TCT TAT GTT AAC CTA CCT ACC ATT GCG CTG
 TGT AAC ACA GAT TCT CCT CTG CGC TAT GTG GAC ATT GCC ATC CCA
 TGC AAC AAC AAG GGA GCT CAC TCA GTG GGT TTG ATG TGG TGG ATG
 CTG GCT CGG GAA GTT CTG CGC ATG CGT GGC ACC ATT TCC CGT GAA
 15 CAC CCA TGG GAG GTC ATG CCT GAT CTG TAC TTC TAC AGA GAT CCT
 GAA GAG ATT GAA AAA GAA GAG CAG GCT GCT GCT GAG AAG GCA GTG
 ACC AAG GAG GAA TTT CAG GGT GAA TGG ACT GCT CCC GCT CCT GAG
 20 TTC ACT GCT ACT CAG CCT GAG GTT GCA GAC TGG TCT GAA GGT GTA
 CAG GTG CCC TCT GTG CCT ATT CAG CAA TTC CCT ACT GAA GAC TGG
 AGC GCT CAG CCT GCC ACG GAA GAC TGG TCT GCA GCT CCC ACT GCT
 CAG GCC ACT GAA TGG GTA GGA GCA ACC ACT GAC TGG TCT TAA GCT
 25 GTT CTT GCA TAG GCT CTT AAG CAG CAT GGA AAA ATG GTT GAT GGA
 AAA TAA ACA TCA GTT TCT.

in whole, or in part.

4. A method according to any preceding Claim, wherein said probe has the ability to hybridise to laminin receptor mRNA.
5. A method according to any preceding Claim, wherein said probe encodes a peptide fragment consisting essentially of a sequence selected from:

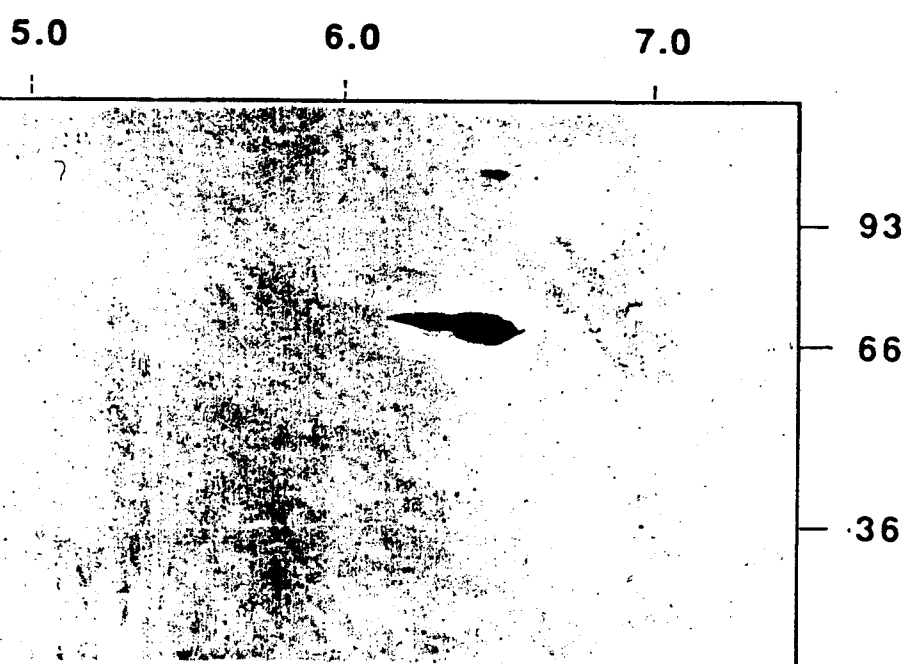
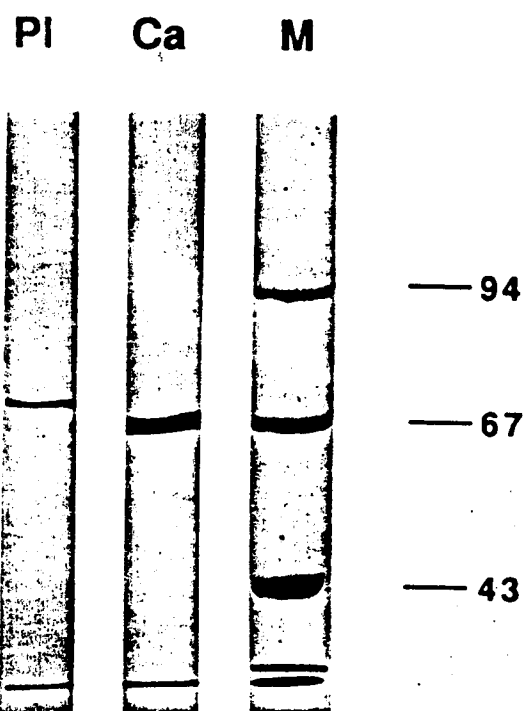
Pro-Thr-Glu-Asp-Trp-Ser-Ala-Gln-Pro-Ala-Thr-Glu-
 Asp-Trp-Ser-Ala-Ala-Pro-Thr-Ala;
 40 Met-leu-Ala-Arg-Glu-Val-Leu-Arg; and
 Leu-Leu-Leu-Ala-Ala-Arg-Ala-Ile-Val-Ala-Ile

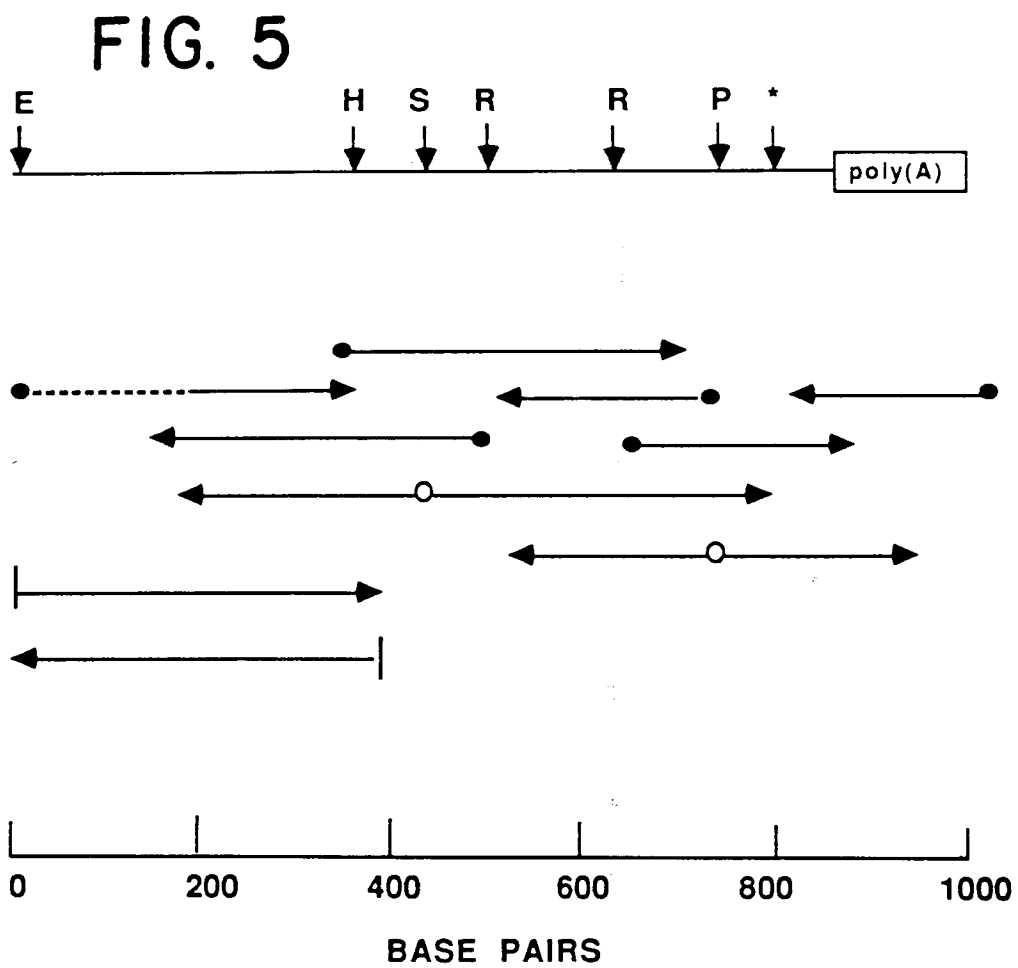
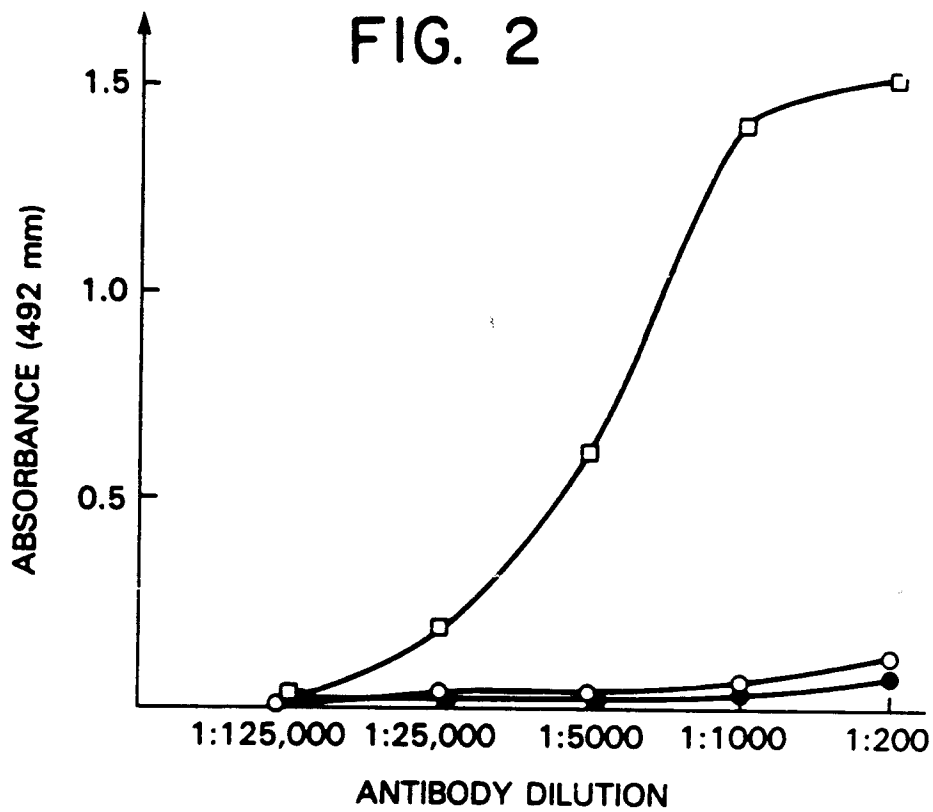
or a fragment thereof capable of binding to laminin, the probe being sufficient to identify a laminin receptor nucleic acid base sequence by Southern blotting.

6. A method according to any preceding Claim, wherein said probe is capable of identifying laminin receptor mRNA or a laminin receptor gene by Southern blotting.
7. A method according to any preceding Claim for diagnosing the aggressiveness of a carcinoma, wherein purified RNA from said carcinoma is reacted with said probe.
8. A method according to any preceding Claim, wherein the probe is detected chromogenically or by radiolabelling.

FIG. 1

Human Laminin Receptor





**SPECIFICITY OF MONOCLONAL ANTIBODY 2H5
FOR λ ELR6 PLAQUES**

FIG. 3-1

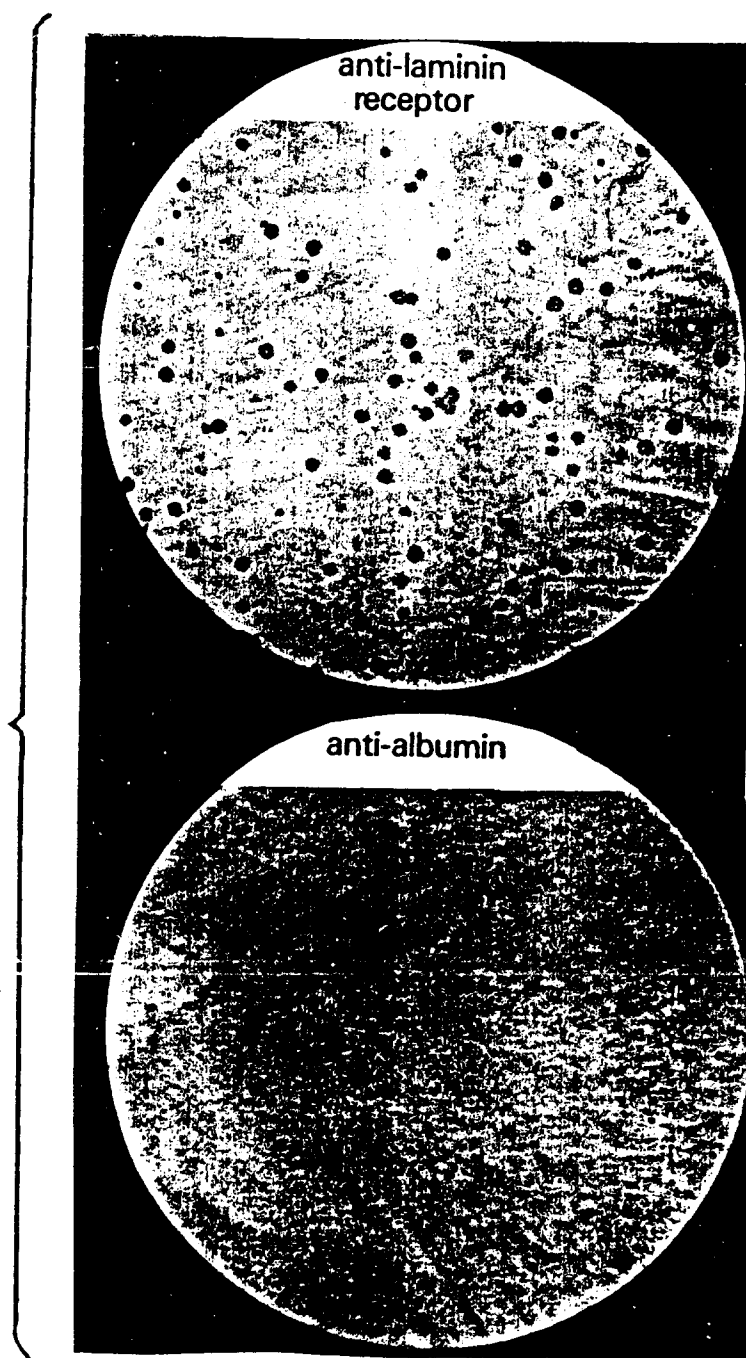


FIG. 3-2

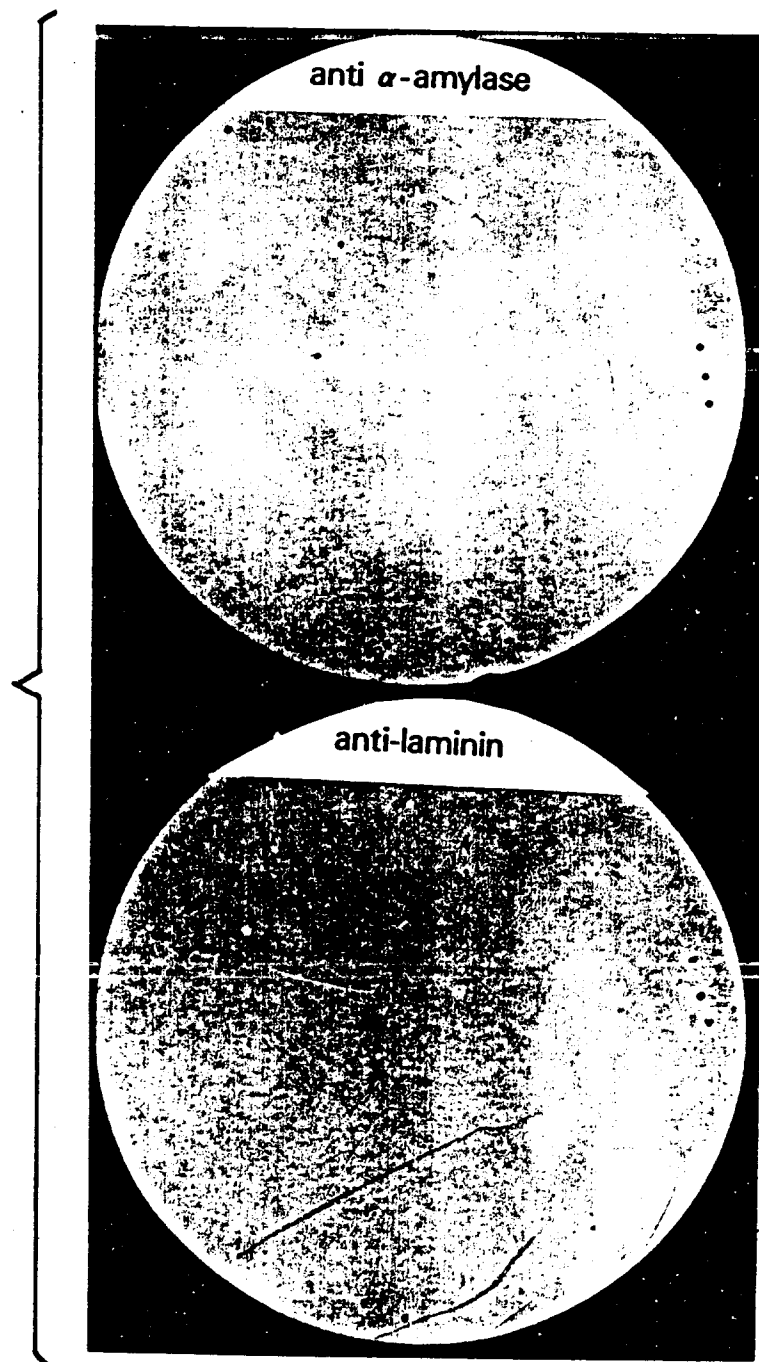
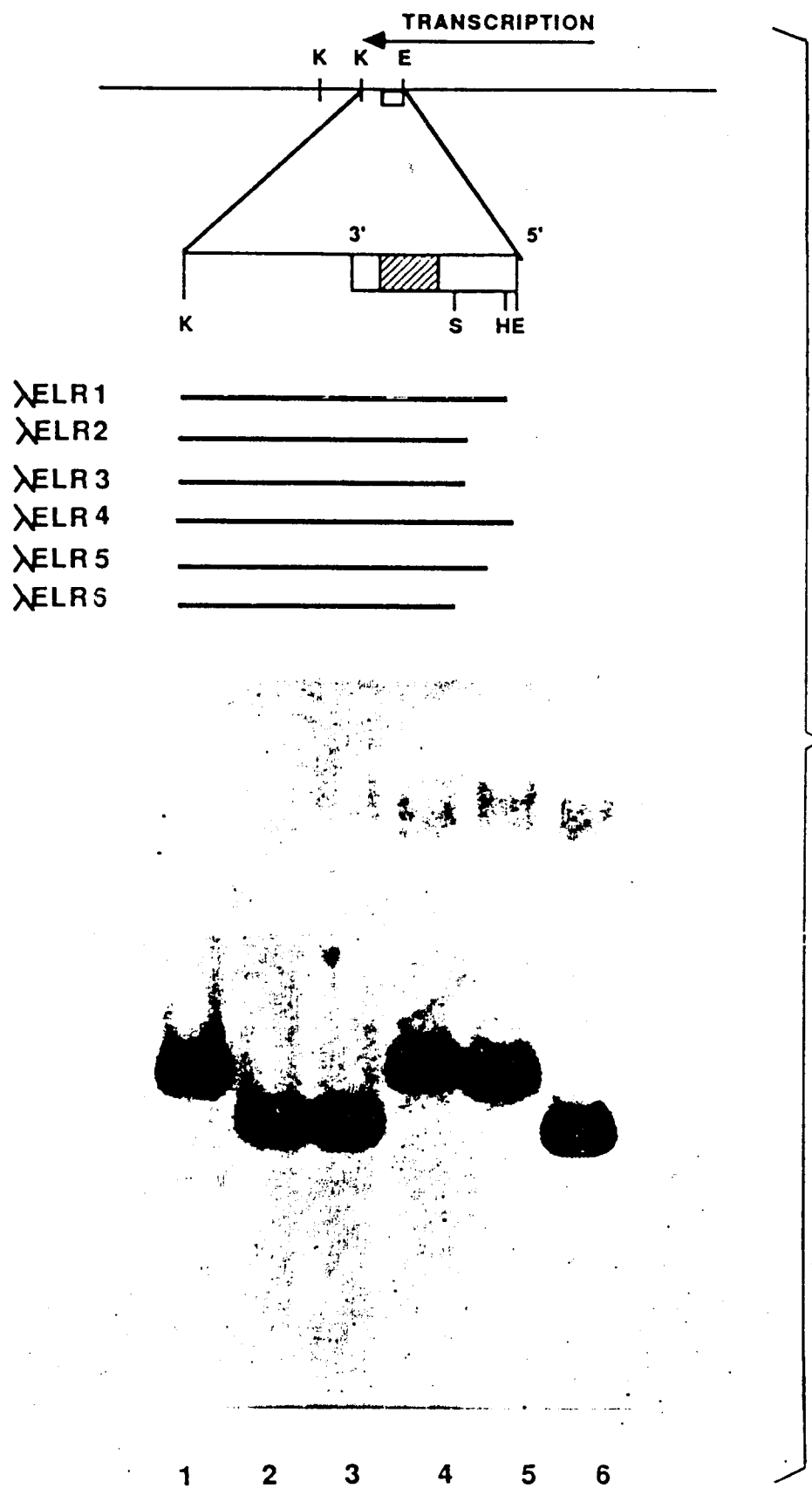


FIG. 4



<u>GAA</u> Eco	<u>TTC</u> RI	CGT arg	GAT asp	GGC gly	ATC ile	TAT tyr	ATC ile	ATA ile	AAT asn	CTC leu
GCA ala	GCT ala	CGT arg	GCA ala	ATT ile	GTT val	GCC ala	ATT ile	GAA glu	AAC asn	CCT pro
AAT asn	ACT thr	GGC gly	CAG gln	AGG arg	GCT ala	GTG val	CTG leu	AAG lys	TTT phe	GCT ala
GGC gly	CGC arg	TTC phe	ACT thr	CCT pro	GGA gly	ACC thr	TTC phe	ACT thr	AAC asn	CAG gln
CTT leu	CTT leu	GTG val	GTT val	ACT thr	GAC asp	CCC pro	AGG arg	GCT thr	GAC asp	CAC his
AAC asn	CTA leu	CCT pro	ACC thr	ATT ile	GCG ala	CTG leu	TGT cys	AAC asn	ACA thr	GAT asp
ATC ile	CCA pro	TGC cys	AAC asn	AAC asn	AAG lys	GGA gly	GCT ala	CAC his	TCA ser	GTG val
GAA glu	GTT val	CTG leu	CGC arg	ATG met	CGT arg	GGC gly	ACC thr	ATT ile	TCC ser	CGT arg
CTG leu	TAC tyr	TTC phe	TAC tyr	AGA arg	GAT asp	CCT pro	GAA glu	GAG glu	ATT ile	GAA glu
GCA ala	GTG val	ACC thr	AAG lys	GAG glu	GAA glu	TTT phe	CAG gln	GGT gly	GAA glu	TGG trp
ACT thr	CAG gln	CCT pro	GAG glu	GTT val	GCA ala	GAC asp	TGG trp	TCT ser	GAA glu	GGT gly
CAA gln	TTC phe	CCT pro	ACT thr	GAA glu	GAC asp	TGG trp	AGC ser	GCT ala	CAG gln	CCT pro
ACT thr	GCT ala	CAG gln	GCC ala	ACT thr	GAA glu	TGG trp	GTA val	GGA gly	GCA ala	ACC thr
TAG	GCT	CTT	AAG	CAG	CAT	GGA	AAA	ATG	GTT	GAT

FIG. 6-1

AAG lys	AGG arg	ACC thr	TGG trp	GAG glu	AAG lys	CTT leu	CTG leu	CTG leu	60
GCT ala	GAT asp	GTC val	AGT ser	GTT val	ATA ile	TCC ser	TCC ser	AGG arg	120
GCT ala	GCC ala	ACT thr	GGA gly	GCC ala	ACT thr	CCA pro	ATT ile	GCT ala	180
ATC ile	CAG gln	GCA ala	GCC ala	TTC phe	CGG arg	AAG lys	CCA pro	CGG arg	240
CAG gln	CCT pro	CTC leu	ACG thr	GAG glu	GCA ala	TCT ser	TAT tyr	GTT val	300
TCT ser	CCT pro	CTG leu	CGC arg	TAT tyr	GTG val	GAC asp	ATT ile	GCC ala	360
GGT gly	TTG leu	ATG met	TGG trp	TGG trp	ATG met	CTG leu	GCT ala	CGG arg	420
GAA glu	CAC his	CCA pro	TGG trp	GAG glu	GTC val	ATG met	CCT pro	GAT asp	480
AAA lys	GAA glu	GAG glu	CAG gln	GCT ala	GCT ala	GCT ala	GAG glu	AAG lys	540
ACT thr	GCT ala	CCC pro	GCT ala	CCT pro	GAG glu	TTC phe	ACT thr	GCT ala	600
GTA val	CAG gln	GTG val	CCC pro	TCT ser	GTG val	CCT pro	ATT ile	CAG gln	660
GCC ala	ACG thr	GAA glu	GAC asp	TGG trp	TCT ser	GCA ala	GCT ala	CCC pro	720
ACT thr	GAC asp	TGG trp	TCT ser	TAA	GCT	GTT	CTT	GCA	780
GGA	AAA	TAA	ACA	TCA	GTT	TCT	A(n)		

FIG. 6-2

FIG. 7

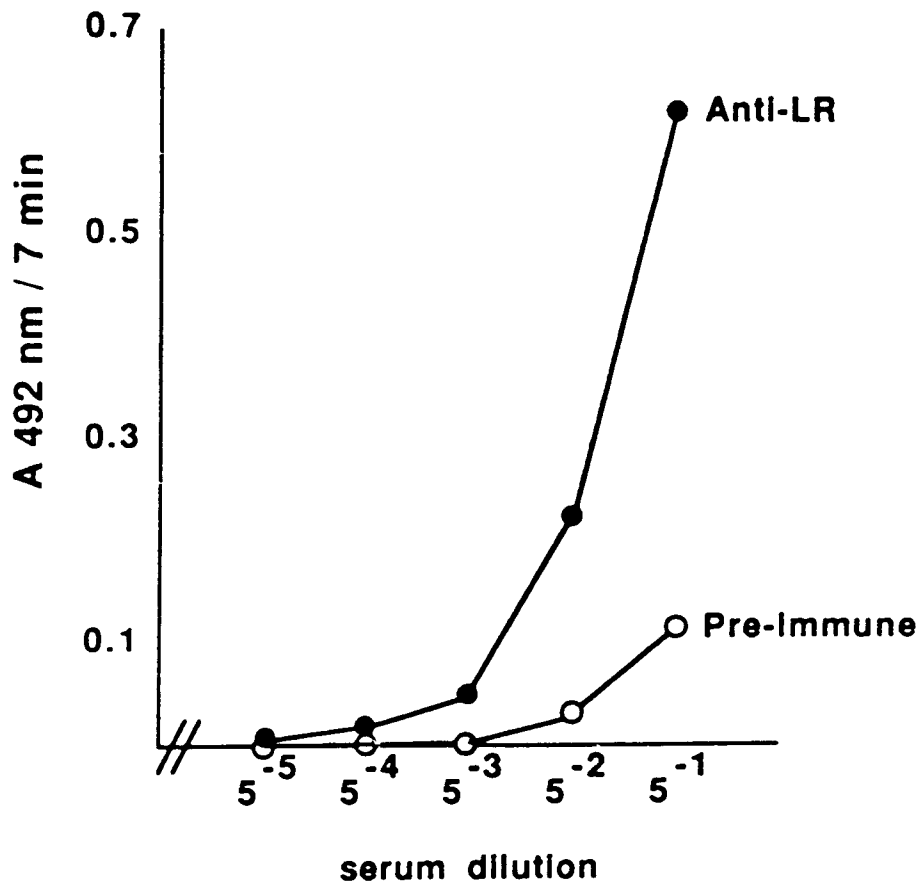


FIG. 9

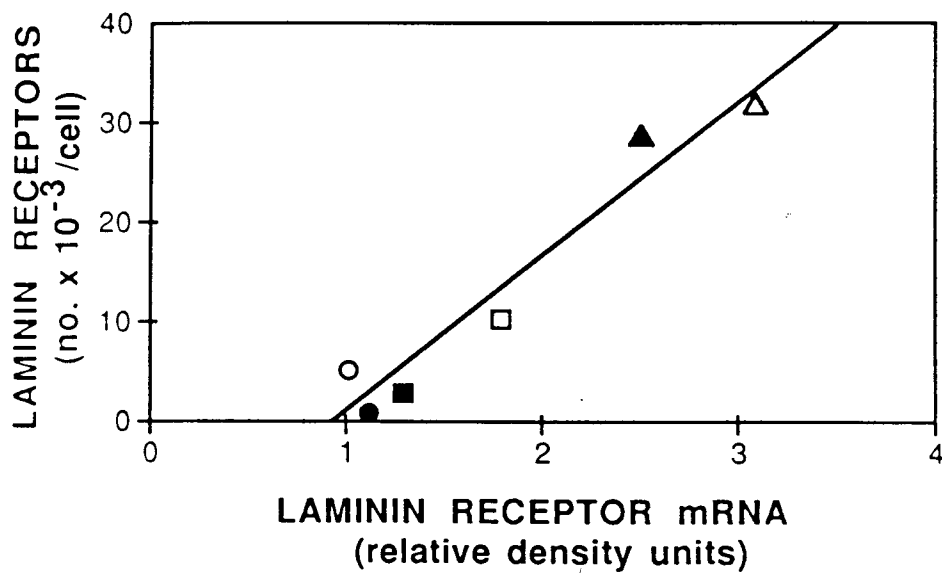
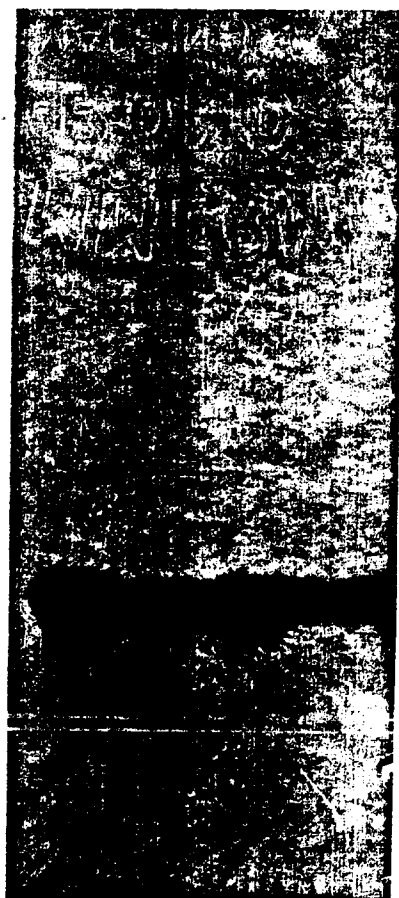
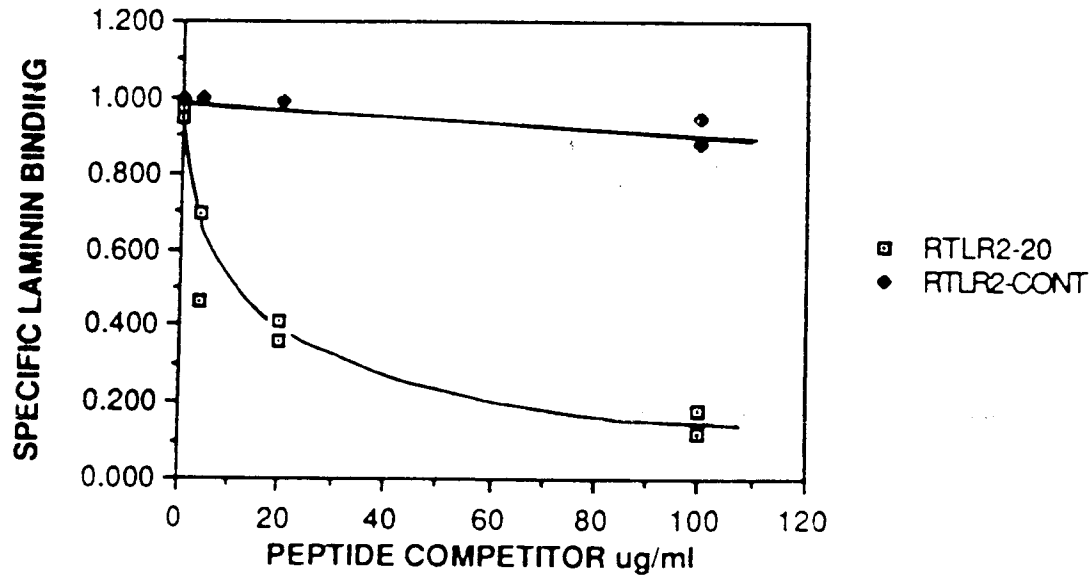
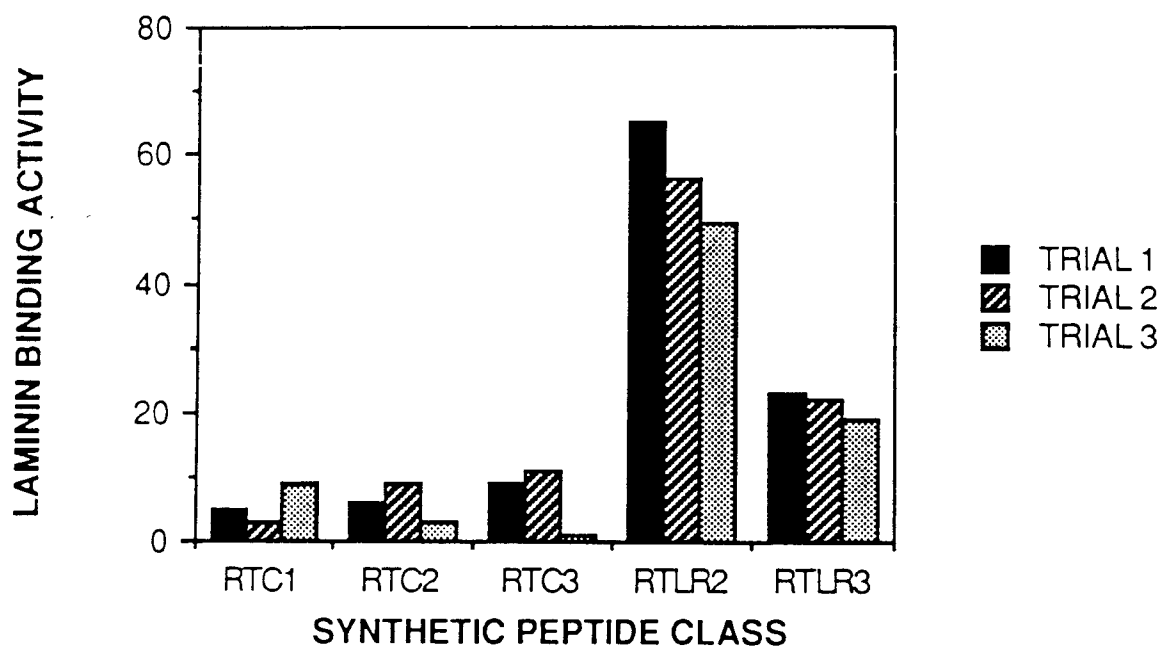


FIG. 8

1700 —



1 2 3 4

FIG. 10**INHIBITION OF LAMININ BINDING TO CELLS****FIG. 11****LAMININ RECEPTOR BINDING DOMAIN**



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 93 10 2626

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	CANCER RESEARCH vol. 45, no. 6, 1985, pages 2713 - 2719 P. HORAN HAND ET AL. 'Expression of laminin receptor in normal and carcinomatous tissues as defined by a monoclonal antibody' ----		C12N15/12 C12Q1/68
A	BIOCHEM. BIOPHYS. RES. COMM. vol. 111, no. 3, 1983, pages 804 - 808 N. RAO ET AL. 'Isolation of a tumor cell laminin receptor' -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C12N C12Q
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 05 APRIL 1993	Examiner SKELLY J.M.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			